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Glycosylation and Processing of High Levels of Active Human Glucocerebrosidase in Invertebrate Cells Using a Baculovirus Expression Vector

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ABSTRACT

A human cDNA containing the complete coding region for the lysosomal glycoprotein glucocerebrosidase (EC 3.2.1.45) was introduced into the genome of *Autographa californica* nuclear polyhedrosis virus downstream from the polyhedrin promoter. Infection of *Spodoptera frugiperda* cells (SF9) with recombinant virus produced high levels of glucocerebrosidase, 40% of which was in the culture medium. The amino-terminal amino acid sequence of the recombinantly produced enzyme was identical to that of mature, human placental glucocerebrosidase, demonstrating that the signal sequence of the human preenzyme was recognized and appropriately removed in the SF9 invertebrate cells. The glucocerebrosidase in both the culture supernatant and SF9 cell pellet was glycosylated and contained, in part, high mannose oligosaccharide. These results demonstrate that insect cells can be used to produce abundant quantities of active mature human glucocerebrosidase that contains high mannose oligosaccharide as a consequence of post-translational processing.

INTRODUCTION

MUTATIONS IN THE GENE for the lysosomal enzyme glucocerebrosidase (EC 3.2.1.45, β-D-glucosyl-*N*-acylsphingosine glucohydrolase) result in the sphingolipidosis called Gaucher disease (Ginns *et al.*, 1984; Tsuji *et al.*, 1987). Although the biochemical defect is present in all tissues, the accumulation of the neutral glycosphingolipid glucosylceramide occurs predominantly within reticuloendothelial cells. On the basis of clinical signs and symptoms, Gaucher disease has been divided into three major phenotypes: type 1, non-neuronopathic; type 2, acute neuronopathic; and type 3, chronic neuronopathic (Barranger and Ginns, in press). Despite the stereotypic presentation of type 2 disease, within the type 1 and type 3 phenotypes there is marked variation in age of symptom onset and severity as well as in organ involvement. To understand more fully the biochemical basis for the heterogeneity seen in the different phenotypes of Gaucher disease, investigators have focused their attention on both biochemical and structural characterizations of normal and mutant glucocerebrosidases (Ginns *et al.*, 1982, 1983; Aerts *et al.*, 1985; Fabbro *et al.*, 1987; Graves *et al.*, 1986).

Although the amino acid sequence of normal human glucocerebrosidase has been determined by both direct chemical sequencing of placental glucocerebrosidase (B. Martin, unpublished data) and from cDNA (Ginns *et al.*, 1984, 1985; Sorge *et al.*, 1985; Tsuji *et al.*, 1986) and genomic DNA (Choudary *et al.*, 1985) deduced amino acid sequences, neither the active site nor other functional domains of even the normal enzyme have been well characterized (Grabowski *et al.*, 1984, 1985b). However, it has been demonstrated that the mutations causing Gaucher disease result in catalytically defective glucocerebrosidases (Brady *et al.*, 1965; Patrick, 1965) that differ from the normal enzyme in stability (Jonsson *et al.*, 1985), post-transla-

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tional processing (Ginns *et al.*, 1982, 1983, 1984; Erickson *et al.*, 1985; Grabowski *et al.*, 1985a; Jonsson *et al.*, 1987), and/or compartmentalization (Willemsen *et al.*, 1987). Although some of these allelic mutations have been documented by sequencing type 1 (Tsuji *et al.*, in press) and type 2 (Tsuji *et al.*, 1987) genomic DNAs for glucocerebrosidase, sufficient quantities of homogeneous mutant enzyme have not been available to carry out extensive structural analyses.

To determine whether sufficient glucocerebrosidase could be produced recombinantly for both structural analyses and *in vivo* therapeutic evaluations, we investigated the feasibility of producing abundant quantities of normal human glucocerebrosidase using a recombinant baculovirus derived from *Autographa californica* nuclear polyhedrosis virus (AcNPV) in eukaryotic insect cells (Smith *et al.*, 1985; Matsuura *et al.*, 1987; Maeda *et al.*, 1985).

Under transcriptional control of the polyhedrin promoter in the recombinant nuclear polyhedrosis virus, the normal human glucocerebrosidase cDNA directed the synthesis of abundant quantities of glycosylated, active enzyme. In this report we compare structural and biochemical properties of the native human placental enzyme with those of the SF9 cell pellet and culture supernatant forms of glucocerebrosidase produced using the baculovirus-derived expression vector.

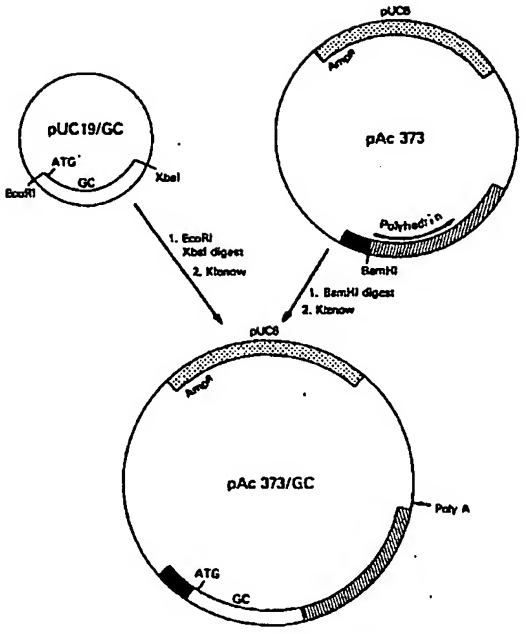

FIG. 1. Construction of the baculovirus-derived vector pAc373/GC containing human glucocerebrosidase cDNA. The *Eco* RI-*Xba* I fragment from pUC19/GC that contained the complete coding region for human glucocerebrosidase was blunted with Klenow polymerase. This insert, containing both the mature enzyme and signal peptide coding sequences, was ligated in a 5′ to 3′ orientation into the *Bam* HI site (also blunted with Klenow) of pAc373 downstream from the polyhedrin promoter.

MATERIALS AND METHODS

Materials

Restriction endonucleases and recombinant enzymes were obtained either from Life Science Technologies or New England Biolabs. Concanavalin A-Sepharose was obtained from Pharmacia. Octyl- and decyl-agarose were purchased from ICN Biomedicals, Inc. Polyvinylidene difluoride (PVDF) membranes, 0.45 μm pore size, were obtained from Millipore Corp. Sequencer chemicals and solvents for on-line PTH analysis were purchased from Applied Biosystems Inc. Endoglycosidase-H was from Miles Scientific while *N*-glycanase was purchased from Genzyme Corp.

Construction of recombinant baculoviruses

Spodoptera frugiperda SF9 cells, plasmid pAc373, and wild-type AcNPV strain E2 were obtained from Max Summers (Texas A & M University). The SF9 cells were maintained in culture at 28°C using TNM-FH media (GIBCO) (Hink, 1970). The cDNA for human glucocerebrosidase was obtained from plasmid pUC19/GC, a derivative of an Okayama–Berg clone from a SV40-transformed human fibroblast cDNA library (Okayama and Berg, 1983). This cDNA contained 5′ and 3′ untranslated sequences as well as the complete coding region for glucocerebrosidase. As shown in Fig. 1, pAc373/GC was generated by ligation of the blunted *Eco* RI-*Xba* I fragment from pUC19/GC into the blunted unique *Bam* HI site of pAC373. Correct orientation of the glucocerebrosidase cDNA insert was determined by restriction endonuclease analysis.

Recombinant baculovirus containing the human glucocerebrosidase coding sequence under transcriptional control of the polyhedrin promoter was produced by cotransfection of wild-type virus, AcNPV, with plasmid pAc373/GC into SF9 cells (Summers and Smith, 1987). Five to six days after cotransfection, virus was harvested from the culture supernatant and used to inoculate new monolayers of SF9 cells in Petri dishes that were subsequently overlaid with 1.5% low-melting agarose containing TNM-FH medium. Seventy-two hours later, the agarose overlay was removed and stored at 4°C, and the cell monolayer was blotted onto a nitrocellulose disk (BA85, Schleicher & Schuell). The disk was hybridized to the random primed ^{32}P-labeled *Eco* RI-*Xba* I glucocerebrosidase cDNA fragment from pUC19/GC (Wahl *et al.*, 1979; Feinberg and Vogelstein, 1983). Areas on the agarose overlay corresponding to points on the nitrocellulose disk showing hybridization signal were excised and placed in 1 ml of TNM-FH medium. This virus was used for infection of SF9

monolayer cultures, and an additional five cycles of infection-hybridization were carried out during the plaque purification.

Enzyme purification

Recombinantly produced glucocerebrosidase was partially purified using a modification of the procedure described by Furbish *et al.* (1977). Cell culture supernatants were precipitated with 195 g/liter ammonium sulfate. SF9 cell pellets containing the recombinantly produced glucocerebrosidase were extracted into 25 m*M* sodium phosphate buffer pH 6.5, containing 150 m*M* NaCl and 0.1% Triton X-100, followed by sonication twice at 50 W for 10 sec. After precipitation with ammonium sulfate (195 g/liter), the resuspended pellets were extracted with *n*-butanol as described, but ultrafiltration using a YM30 membrane (Amicon) replaced dialysis. After decyl- and octyl-agarose hydrophobic interaction chromatography at room temperature, the fractions containing glucocerebrosidase activity were pooled, and the ethylene glycol concentration was reduced using an Amicon ultrafiltration cell fitted with a YM30 membrane.

Carbohydrate characterization

Endoglycosidase-H was dissolved in 100 m*M* sodium acetate pH 6.0 at a final concentration of 10 U/ml. *N*-Glycanase was supplied as a 250 U/ml suspension in 50% glycerol. Either human placental enzyme or 50-μl aliquots of the decyl-agarose fractions containing glucocerebrosidase activity were adjusted to 0.5% $NaDodSO_4$/1 *M* β-mercaptoethanol and boiled for 2 min. The samples were then diluted with appropriate buffer to either 200 m*M* sodium acetate pH 6.0 (for endoglycosidase-H) or 200 m*M* sodium phosphate pH 8.5 (for *N*-glycanase) and a final composition of 0.1% $NaDodSO_4$, 0.7% NP-40, and 0.02 *M* β-mercaptoethanol (Trimble and Maley, 1984; Tarentino *et al.*, 1985). The samples were again boiled for 1 min, and then endoglycosidase-H and *N*-glycanase were added to final concentrations of 50 mU/ml and 20 U/ml, respectively. Digestions were for 16 h at 37°C. Carboxypeptidase Y was used as a control for both deglycosylation reactions.

Western blot analysis

$NaDodSO_4$-polyacrylamide gel electrophoresis and Western blot analysis were performed as previously described by Ginns *et al.* (1982).

Amino acid sequence analysis

Samples used for amino acid sequence analysis were electrophoretically fractionated on $NaDodSO_4$ polyacrylamide gels as described above and then transferred to PVDF membranes as described by Matsudaira (1987). Briefly, after electrophoresis, the gel was incubated in transfer buffer (0.01 *M* CAPS, 10% methanol, pH 11.0) for 10 min prior to transblotting (50 mA for 4 hr). The gel was then washed with HPLC-grade water for 5 min, stained with 0.1% Coomassie Blue R250 (in 50% methanol) for 5 min, and finally destained for 10 min with 50% methanol–10% acetic acid. The PVDF membrane was again washed with HPLC-grade water, dried under a stream of nitrogen, and stored in a sealed bag at −20°C until used for amino acid sequencing.

Amino acid sequence analysis was accomplished using an Applied Biosystems Model 470A gas-phase sequencer equipped with a Model 120A on-line PTH-amino acid analyzer. The program 03R PTH was used directly for sequencing without pretreatment of the membrane strip with Polybrene. An approximately 2 × 8 mm piece of PVDF membrane containing the protein band of interest (Fig. 4, below) was excised, centered on the Teflon seal, and placed in the cartridge block of the sequencer. Multiple strips of the PVDF membrane could be stacked in this manner, thus increasing the amount of protein available for sequencing. The initial and repetitive yields for sequencing recombinant glucocerebrosidase were calculated by comparison with the yields obtained after 100 pmoles of human placental glucocerebrosidase were electrophoresed, transblotted to PVDF, and subjected to 10 cycles of amino acid sequence (Table 1).

Glucocerebrosidase assays

For pH profile and inhibition experiments, glucocerebrosidase activity was measured using 100 m*M* potassium phosphate buffer containing 0.15% Triton X-100, 2.5 μl of β-D-[1-^{14}C]glucocerebroside (7.5 mg/ml in sodium taurocholate at 50 mg/ml), and sample in a total volume of 200 μl (Ginns *et al.*, 1982). Preincubations with conduritol-B-epoxide were for 30 min at 37°C (Legler, 1977). For K_m determination, β-glucosidase activity was assayed at pH 5.9 using the artificial substrate 4-methylumbelliferyl-β-D-glucopyranoside (4MUGP) in 100 m*M* potassium phosphate buffer containing 0.15% Triton X-100 and 0.125% sodium taurocholate (Peters *et al.*, 1976; Wenger *et al.*, 1978). Purification of recombinant glucocerebrosidase was also monitored using 4MUGP.

RESULTS

Expression of human glucocerebrosidase in SF9 cells

Cotransfection of SF9 cells with pAc373/GC (Fig. 1) and wild-type AcNPV DNA using a modification of the calcium phosphate precipitation technique (Graham and van der Eb, 1973) generated recombinant baculovirus that contained human glucocerebrosidase cDNA. As early as 5 days after the initial infection, Western blot analysis of culture medium demonstrated the presence of cross-reac-

TABLE 1. REPETITIVE YIELD OF RECOMBINANT AND PLACENTAL HUMAN GLUCOCEREBROSIDASE DURING SEQUENCE ANALYSIS

	A	R	P	–	I	P	K	S	F	G	Repetitive yield[a] (%)
					Initial yield 77%						
Amt.[b] (pmole): placental enzyme	77	14	49	–	43	31	23	11	40	45	92
Amt.[b] (pmole): enzyme in media	14	7	14	–	9	10	7	2	10	10	90
Amt.[b] (pmole): enzyme in cell pellet	10	2	6	–	3	4	1	tr	3	2	90

[a]Repetitive yield was calculated from P3 to P6..
[b]Amount transblotted was estimated by comparison to the intensity of bands obtained by transfer of 100 pmoles of human placental glucocerebrosidase (media 18 pmoles; cell pellet 13 pmoles).

tive material (CRM) to human glucocerebrosidase (data not shown) that was similar to the pattern shown in Fig. 2, lane M4. Recombinant baculovirus was subsequently plaque-purified as described above and used to infect SF9 invertebrate cells. Seventy-two hours postinfection, the SF9 cell pellets and culture medium were harvested. Western blot analysis of both recombinantly produced enzyme following decyl-agarose hydrophobic chromatography and purified human placental glucocerebrosidase is shown in Fig. 2, lanes P1, M4, and C7. Prominent bands of cross-reactive material to human glucocerebrosidase were seen at 59 kD in the culture medium (Fig. 2, M4) and at 65 kD in the cell pellet extract (Fig. 2, C7) lanes. Both the cell pellet and culture medium-associated glucocerebrosidase CRM showed a minor band at 52 kD. In contrast, human placental glucocerebrosidase CRM showed a major band at 65 kD. These apparent molecular weights for recombinant human glucocerebrosidase are in good agreement with those previously reported from Western blot (Ginns *et al.*, 1982) and pulse-chase (Erickson *et al.*, 1985; Jonsson *et al.*, 1987) analyses of human tissues and cell lines. Western blot analysis of SF9 cells infected only with the wild-type baculovirus did not show any CRM to human glucocerebrosidase.

Recombinant glucocerebrosidase is active in SF9 cells.

The eukaryotic SF9 cells infected with recombinant baculovirus and the culture medium were harvested 72 h postinfection. From 1.2×10^8 SF9 cells infected with recombinant baculovirus, the cell extract and culture medium (200 ml) contained 400,000 units and 270,000 units of glucocerebrosidase activity, respectively. After the decyl-agarose hydrophobic chromatography step in the purification procedure, from 15 to 40% of this initial enzyme activity was recovered. The invertebrate eukaryotic SF9 cells infected with only wild-type baculovirus showed no significant glucocerebrosidase activity, consistent with the absence of enzyme CRM.

The pH profiles of human placental and recombinantly produced human glucocerebrosidases are shown in Fig. 3. In all three cases, peaks of activity are observed between pH 4.5 and 5.0 and pH 5.5 and 6.0. The K_m values for the recombinant glucocerebrosidase from the cell pellet and culture supernatant were 3.6 m*M* and 3.3 m*M*, respectively. After 30 min of incubation at 37°C with the specific catalytic site inhibitor conduritol-B-epoxide, the placental as well as recombinant cell pellet and culture supernatant glucocerebrosidase activities were greater than 95% inhibited at both pH 4.5 and pH 5.5.

Glycosylation of glucocerebrosidase in SF9 cells

Since the post-translational processing of human glucocerebrosidase normally involves carbohydrate modification (Takasaki *et al.*, 1984; Erickson *et al.*, 1985; Furbish *et al.*, 1981; Aerts *et al.*, 1986; Murray *et al.*, 1985), we investigated the pattern of glycosylation in SF9 cells. All recombinantly produced glucocerebrosidase activity in both the SF9 cell pellet and culture medium bound to Concanavalin A-Sepharose at 4°C, and could be eluted with α-methyl-mannoside at 37°C (data not shown).

Treatment of either human placental enzyme or the recombinant glucocerebrosidase in the culture medium or cell pellet (Fig. 2, lanes P2, M5, and C8, respectively) with endoglycosidase-H resulted in minor changes in the CRM pattern. However, incubation of the placental or recombinant glucocerebrosidases (Fig. 2, lanes P3, M6, and C9, re-

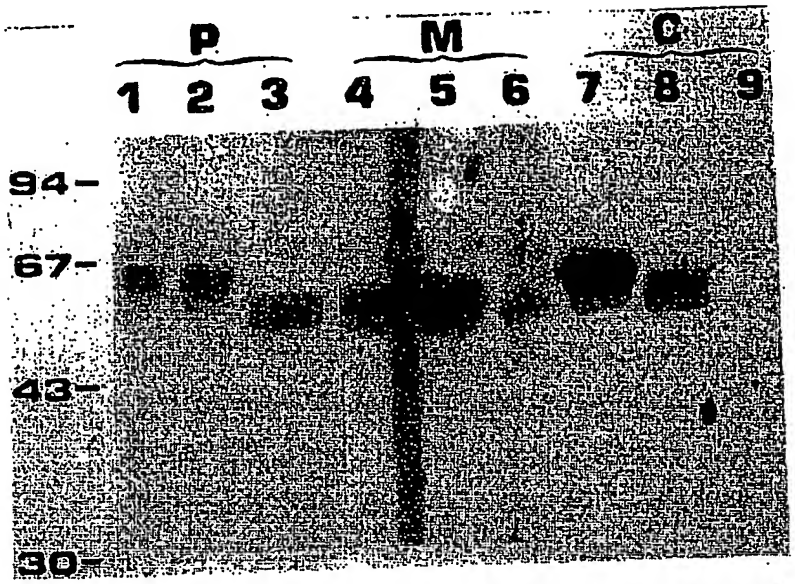

FIG. 2. Immunoblot detection of the recombinant baculovirus-encoded human glucocerebrosidase in infected SF9 cells. Sample preparation, electrophoresis, and Western blot analysis were performed as described in the text. Molecular weight size markers (M_r X10^{-3}) were phosphorylase b, albumin, ovalbumin, and carbonic anhydrase. P, Placental enzyme; M, media; and C, cell-associated. Western blots from untreated placental enzyme or recombinantly produced protein are shown in lanes P1, M4, and C7. Cross-reactive material (CRM) from endoglycosidase-H- and *N*-glycanase-digested samples are shown in lanes P2, M5, C8, and P3, M6, C9, respectively.

spectively) with *N*-glycanase produced a dramatic decrease in the apparent molecular weights of all three species, with the major CRM form appearing at approximately 52 kD in each case.

Sequence analysis of recombinant glucocerebrosidase

To determine whether the amino-terminal processing of recombinant glucocerebrosidase in the invertebrate eukaryotic SF9 cells is identical to that in human tissue, we sequenced the recombinant enzyme. Fractions from the decyl-agarose hydrophobic column that contained glucocerebrosidase activity were pooled, electrophoretically fractionated on polyacrylamide gels, and transblotted to PVDF membranes as described above. As seen from the Coomassie-stained PVDF membrane (Fig. 4, lanes 1 and 2) only two major protein bands were present. Western blot analysis confirmed that only the lower band contained CRM to human glucocerebrosidase (data not shown). Typically, approximately 2 μg of total protein per gel lane transferred to the PVDF membrane. The results obtained from sequencing placental and recombinant glucocerebrosidases are shown in Fig. 5. Each sample gave a single unique amino acid sequence. The amino-terminal sequence of recombinant glucocerebrosidases from the SF9 cell pellet and culture medium was identical to that of the purified human placental enzyme. The blank in position 4 of the recombinant enzyme sequences is consistent with cysteine because a cysteine at this position in the purified placental enzyme could only be identified after reduction and alkylation. The repetitive yields are shown in Table 1. From these data it is clear that the signal peptide of the preenzyme was correctly removed from the recombinant glucocerebrosidase produced in the invertebrate insect cells.

DISCUSSION

Our results demonstrate that high levels of active human glucocerebrosidase can be synthesized by the invertebrate SF9 cell-baculovirus expression system. It has been shown previously that the maturation of human glucocerebrosidase involves removal of a signal peptide (Ginns *et al.*, 1985) followed by carbohydrate processing that involves the conversion of high mannose to complex oligosaccharide chains (Erickson *et al.*, 1985). Microsequence analysis of recombinant glucocerebrosidase from both the SF9 insect cell pellet and culture supernatant demonstrated that the amino-terminal sequence was identical to that of human placental glucocerebrosidase (Tsuji *et al.*, 1986). Importantly, the glycoprotein-processing mechanisms within the insect cells were able to recognize and appropriately cleave the mammalian signal peptide of glucocerebrosidase and produce a mature glycosylated protein having an

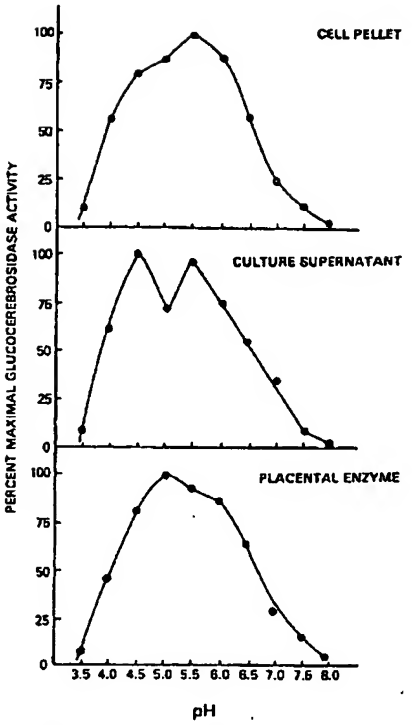

FIG. 3. Comparison of pH profiles of human placental glucocerebrosidase to recombinantly produced enzyme. Assays were performed using [^{14}C]glucocerebroside as described in the text.

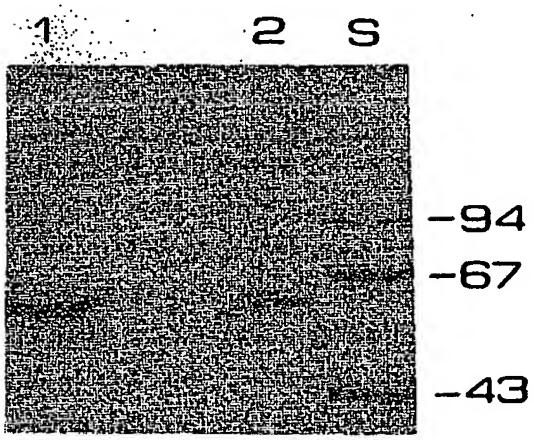

FIG. 4. Coomassie stain of polyvinylidine difluoride membrane (PVDF) containing electroblotted protein. Three micrograms of phosphorylase b, 4 μg of albumin, and 5 μg of ovalbumin were used as standards (lane S). Approximately 1 μg of partially purified recombinant glucocerebrosidase from infected SF9 cell pellets (lanes 1 and 2) was fractionated electrophoretically, transferred to the PVDF membrane, and stained with Coomassie, and amino acid sequence was determined as described.

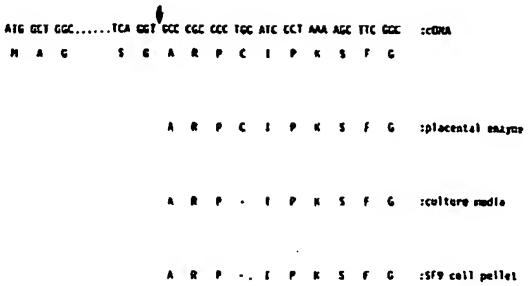

FIG. 5. Amino-terminal sequence of recombinant glucocerebrosidase in the SF9 cell pellet and culture supernatant. The mammalian peptidase cleavage site is indicated by the arrow. In the recombinant sequences, the blank (-) at position 4 is consistent with the presence of cysteine, since this amino acid was seen in the placental enzyme sequence only after reduction and alkylation of the sample.

amino-terminal sequence identical to that of human placental glucocerebrosidase.

Although the major bands of CRM to human glucocerebrosidase in the culture medium and cell pellet had apparent molecular weights of 59 kD and 65 kD (Fig. 2), respectively, both contained a minor band of CRM at 52 kD. The molecular weight of this minor band is consistent with the previously reported size of the unglycosylated form of human glucocerebrosidase, suggesting that a small proportion of the recombinantly produced enzyme does not contain carbohydrate (Erickson *et al.*, 1985).

Previously, it had been demonstrated that glycosylation of human glucocerebrosidase and other glycoproteins in heterologous cells is species specific (Sheares *et al.*, 1986; Choudary *et al.*, 1986c). Therefore, it was reasonable to expect that the pattern of glucocerebrosidase CRM in SF9 invertebrate cells might not be identical to that seen in human tissues. Treatment of recombinantly produced enzyme with *N*-glycanase produced a single form at 52 kD (Fig. 2, lanes M6 and C9), confirming the presence of *N*-linked carbohydrate. The changes in the molecular weight of glucocerebrosidase CRM after endoglycosidase-H digestion (Fig. 2, lanes M5 and C8) indicate that human glucocerebrosidase produced in these invertebrate cells has at least some high-mannose oligosaccharide chains. These results also suggest that after the removal of the signal peptide, the post-translational processing of the recombinantly produced glucocerebrosidase in SF9 insect cells is similar to that in human tissues, and only involves carbohydrate modification (Erickson *et al.*, 1985).

The pH profile of human glucocerebrosidase synthesized in insect cells using the baculovirus expression vector is very similar to that reported for glucocerebrosidase isolated from human tissues (Fig. 3) (Aerts *et al.*, 1985). The glucocerebrosidase in the cell pellet and culture supernatant had K_m values of 3.6 m*M* and 3.3 m*M*, respectively,

close to that reported for normal human spleen glucocerebrosidase (K_m = 8 m*M*) (Basu *et al.*, 1984). The recombinantly produced glucocerebrosidase was also appropriately inhibited by conduritol-B-epoxide, a specific active site inhibitor (Legler, 1977; Grabowski *et al.*, 1985b). Based on a specific activity of 1.6×10^4 U/mg for purified human placental glucocerebrosidase, approximately 2.2 mg of active recombinant enzyme could be produced in 2 days from 1 liter of insect cell–recombinant baculovirus culture (6.0×10^8 cells).

Although a number of expression systems might be tried for production of large quantities of proteins (Choudary *et al.*, 1986a, 1986b; Reiner *et al.*, 1987), the structure and biological activity of the recombinant gene product should be as close as possible if not identical to the natural protein. Changes in post-translational modifications due to either the host cell processing machinery or to *in vitro* mutagenized cDNA changes could result in either increased or decreased biological function. This report demonstrates that the invertebrate baculovirus-SF9 cell expression system appropriately processes the mammalian signal sequence of human glucocerebrosidase, produces enzyme that is *N*-glycosylated, and can be used to rapidly produce large quantities of active human glucocerebrosidase.

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